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# FGFR1 and PROKR2 rare variants found in patients with combined pituitary hormone deficiencies

Fernanda A Correa<sup>1</sup>, Ericka B Trarbach<sup>1</sup>, Cintia Tusset<sup>1</sup>, Ana Claudia Latronico<sup>1</sup>, Luciana R Montenegro<sup>1</sup>, Luciani R Carvalho<sup>1</sup>, Marcela M Franca<sup>1</sup>, Aline P Otto<sup>1</sup>, Everlayny F Costalonga<sup>1</sup>, Vinicius N Brito<sup>1</sup>, Ana Paula Abreu<sup>4</sup>, Mirian Y Nishi<sup>1</sup>, Alexander A L Jorge<sup>2</sup>, Ivo J P Arnhold<sup>1</sup>, Yisrael Sidis<sup>3</sup>, Nelly Pitteloud<sup>3</sup> and Berenice B Mendonça<sup>1</sup>

<sup>1</sup>Unidade de Endocrinologia do Desenvolvimento, Laboratório de Hormônios e Genética Molecular LIM42, and

<sup>2</sup>Unidade de Endocrinologia Genética, Laboratório de Endocrinologia Celular e Molecular LIM25, Hospital das Clínicas, Disciplina de Endocrinologia, Faculdade de Medicina da Universidade de São Paulo, Av. Dr. Eneas de Carvalho Aguiar, 255, 05403-000 São Paulo, Brazil

<sup>3</sup>Centre Hospitalier Universitaire Vaudois (CHUV), Faculté de Biologie et Médecine de l'Université de Lausanne, Lausanne, Switzerland

<sup>4</sup>Division of Endocrinology, Diabetes, and Hypertension, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA

Correspondence should be addressed to F A Correa

**Email**  
fernandacorrea@usp.br

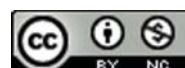
## Abstract

The genetic aetiology of congenital hypopituitarism (CH) is not entirely elucidated. *FGFR1* and *PROKR2* loss-of-function mutations are classically involved in hypogonadotrophic hypogonadism (HH), however, due to the clinical and genetic overlap of HH and CH; these genes may also be involved in the pathogenesis of CH. Using a candidate gene approach, we screened 156 Brazilian patients with combined pituitary hormone deficiencies (CPHD) for loss-of-function mutations in *FGFR1* and *PROKR2*. We identified three *FGFR1* variants (p.Arg448Trp, p.Ser107Leu and p.Pro772Ser) in four unrelated patients (two males) and two *PROKR2* variants (p.Arg85Cys and p.Arg248Glu) in two unrelated female patients. Five of the six patients harbouring the variants had a first-degree relative that was an unaffected carrier of it. Results of functional studies indicated that the new *FGFR1* variant p.Arg448Trp is a loss-of-function variant, while p.Ser107Leu and p.Pro772Ser present signalling activity similar to the wild-type form. Regarding *PROKR2* variants, results from previous functional studies indicated that p.Arg85Cys moderately compromises receptor signalling through both MAPK and Ca<sup>2+</sup> pathways while p.Arg248Glu decreases calcium mobilization but has normal MAPK activity. The presence of loss-of-function variants of *FGFR1* and *PROKR2* in our patients with CPHD is indicative of an adjuvant and/or modifier effect of these rare variants on the phenotype. The presence of the same variants in unaffected relatives implies that they cannot solely cause the phenotype. Other associated genetic and/or environmental modifiers may play a role in the aetiology of this condition.

## Key Words

- ▶ combined pituitary hormone deficiencies
- ▶ *FGFR1*
- ▶ *PROKR2*
- ▶ hypopituitarism

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## Introduction

Combined pituitary hormone deficiencies (CPHD) are defined as the deficiency of two or more pituitary hormones. They can be associated with complex phenotypes such as cranial/facial midline defects and other extra-pituitary features. Mutations in transcription factors such as *PROP1*, *POU1F1*, *GLI2*, *HESX1*, *LHX3*, *LHX4*, *SOX2*, *SOX3* and *OTX2* underlie CPHD (1, 2). There is clinical and genetic overlap between CPHD and isolated hypogonadotrophic hypogonadism (IHH) and/or Kallmann syndrome (KS), such as midline cerebral and facial defects (3). The adenohypophyseal and olfactory placodes share a common embryological origin as they both emerge from the preplacodal field, which could explain this overlap (4).

FGFR1, a tyrosine kinase receptor, is expressed in Rathke's pouch and ventral diencephalon in the developing human embryo (5). PROKR2 is a G protein-coupled receptor essential for normal olfactory bulb development and sexual maturation in mice (6), and it is also involved in angiogenesis and neuronal migration (7).

Loss-of-function mutations in *FGFR1* and *PROKR2* are classically associated with IHH and/or KS (8, 9, 10, 11, 12). Furthermore, mutations in *FGFR1*, *PROKR2* and *FGF8*, the *FGFR1* ligand, have already been found to be associated with hypothalamus–pituitary dysfunction (3, 5, 13).

Our aim was to screen a large cohort of Brazilian patients with CPHD for loss-of-function mutations in *FGFR1* and *PROKR2* to investigate the role of these genes in the aetiology of hypopituitarism.

## Subjects and methods

### Selection of patients

We studied 156 Brazilian patients with CPHD recruited consecutively from the Hospital das Clinicas, University of Sao Paulo Medical School after approval of the ethical committee. In addition, informed written consent was obtained from patients and/or parents. The clinical and radiological features of this cohort are detailed in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article.

The diagnosis of CPHD was based on the failure to have a normal response to a combined pituitary stimulation test (0.05–0.1 U/kg insulin, 200 µg thyrotrophin-releasing hormone and 100 µg gonadotrophin-releasing hormone, i.v.) and/or low basal insulin-like growth factor 1 (IGF1), IGF-binding protein 3, free thyroxine, luteinizing hormone (LH), follicle-stimulating hormone (FSH), oestradiol or

testosterone and cortisol levels. Height was measured with a stadiometer, and height standard deviation was calculated using British references (14). Magnetic resonance imaging (MRI) scans were performed in a 1.5 Tesla unit (Sigma; GE, Milwaukee, WI, USA) using T1- and T2-weighted sagittal and coronal scans. The control group consisted of 400 healthy Brazilian adults.

### Genetic analyses

Genomic DNA was extracted from peripheral blood leucocytes by standard techniques. The entire coding region and exon–intron junctions of the *FGFR1* (ENST00000341462) and *PROKR2* (ENST00000217270) genes were amplified by PCR and sequenced, using primers and conditions described previously (8, 12). All mutations were confirmed in two separate PCR. The variants in DNA and protein sequences are presented according to HGVS nomenclature recommendations ([www.hgvs.org/](http://www.hgvs.org/)). Mutations in *GLI2*, *LHX4*, *HESX1*, *OTX2* and *SOX3* were searched for in the patients harbouring *FGFR1* and *PROKR2* variants.

### In silico analysis

Computational analysis using algorithms from web-based tools such as Mutation Taster (<http://www.mutationtaster.org/>), SIFT (<http://sift.jcvi.org/>) and Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2/>) was performed for prediction of the pathological effects of the substitutions on protein sequence.

### Functional studies

**Luciferase reporter assays** ► Signalling activity of FGFR1 mutants was assessed using L6 myoblast cells as described previously (15). In brief, cells were transiently transfected with WT or altered FGFR1c expression vector in combination with the osteocalcin FGF response element luciferase reporter. Cells were treated with increasing doses of FGF2 and assayed for luciferase activity. The data were plotted and fitted with three-parameter sigmoidal dose–response curves using Prism Software (version 5; GraphPad, La Jolla, CA, USA). Transfection experiments were performed in triplicate and repeated three times. Results of individual experiments were expressed as percentages of the WT value, and the calculated mean maximal activity (top of the curve)

from three independent experiments was compared using Prism's F-test function.

### Receptor expression and maturation studies ►

Endoglycosidase and western analysis were performed as described previously (15). In brief, COS-7 cells were transiently transfected with Myc-tagged WT or mutated *FGFR1* cDNA. Samples of cleared cell lysate (4 µl, approximately 5 µg of total protein) were subjected to PNGaseF and EndoH digestion (New England Biolabs, Ipswich, MA, USA), resolved on NuPAGE 3–8% Tris–acetate gels (Invitrogen) and then subjected to western analysis using an anti-Myc primary antibody (clone 4A6, 1:2000; Upstate Biotechnology, Inc., Lake Placid, NY, USA) and a goat anti-mouse HRP-conjugated secondary antibody (1:20 000; Upstate Biotechnology, Inc.). To control for equal loading, blots were stripped and reprobed with HRP-conjugated anti-β-actin antibody (1:20 000; Abcam, Cambridge, UK). *FGFR1* and β-actin immunoreactivity was quantified by densitometry of film (BioMAX, Kodak) acquired images using ImageJ Software (Bethesda, MD, USA). Overall expression levels of WT and mutant receptors were determined from the PNGase-treated samples and were normalized to their respective β-actin levels. Results were expressed as the ratio between mutant and WT levels. For receptor maturation studies, the upper (mature) and lower (immature) band densities were determined individually from the EndoH-treated samples, and the percentage of the mature fraction was calculated and expressed as a ratio of mutant to WT expression. Experiments were repeated three times, and protein expression and maturation levels were compared between mutant and WT proteins using Student's *t*-test. Data are presented as the mean ± S.E.M.

**Cell surface expression ►** Expression of WT or mutant *FGFR1* at the cell surface was quantified using COS-7 cells and an antibody binding assay (15). Experiments were performed in quadruplicate and repeated four times.

Specific cell-surface expression of altered *FGFR1* proteins was compared with that of the WT protein using Student's *t*-test. Data are presented as the mean ± S.E.M.

## Results

### Genetic analyses

We searched for variants in *GLI2*, *LHX4*, *HESX1*, *OTX2* and *SOX3* genes in the patients harbouring *FGFR1* and *PROKR2* variants. These genes are known to be involved in CPHD and no pathological mutations were found.

**FGFR1 ►** Three missense variants, p.Ser107Leu, p.Arg448Trp and p.Pro772Ser, were identified in a heterozygous state in four unrelated patients (two males), the children of non-consanguineous parents (Table 1). Results of *in silico* analysis indicated that p.Arg448Trp is deleterious according to the Mutation Taster, SIFT and Polyphen-2 tools, whereas p.Ser107Leu and p.Pro772Ser were predicted to be disease-causing only by the Mutation Taster tool.

**PROKR2 ►** Two missense variants, p.Arg85Cys and p.Arg248Glu, were found in a heterozygous state in two unrelated females, the children of non-consanguineous parents (Table 1). Results of *in silico* analysis indicated that p.Arg85Cys is damaging according to Mutation Taster, SIFT and Polyphen-2 whereas p.Arg248Glu was predicted to be benign using the three *in silico* prediction tools.

All the five variants have been detected previously in patients with congenital HH (9, 16, 17). The *FGFR1* p.Pro772Ser variant was found in five out of 400 (1.25%) Brazilian healthy controls, and the *PROKR2* variant p.Arg85Cys was found in one out of 400 (0.25%). The other three variants were not identified in Brazilian healthy controls.

Regarding population genetics, the *FGFR1* variant p.Ser107Leu has a minor allele frequency (MAF) of 0.6%

**Table 1** Genotypes and functional *in silico* prediction of *FGFR1* and *PROKR2* variants found in patients.

Patient	Gene	Nucleotide change	Protein change	Functional domain	In silico prediction tools		
					Mutation Taster	SIFT	Polyphen-2
I	<i>FGFR1</i>	c.320C>T	p.Ser107Leu	Ig-like C2-type 1 domain	Disease-causing	Tolerated	Benign
II	<i>FGFR1</i>	c.1342C>T	p.Arg448Trp	Juxta-membrane domain	Disease-causing	Affects protein function	Probably damaging
III	<i>FGFR1</i>	c.2314C>T	p.Pro772Ser	C-terminal tail	Disease-causing	Tolerated	Benign
IV	<i>FGFR1</i>	c.2314C>T	p.Pro772Ser	C-terminal tail	Disease-causing	Tolerated	Benign
V	<i>PROKR2</i>	c.253C>T	p.Arg85Cys	First intracellular loop	Disease-causing	Affects protein function	Probably damaging
VI	<i>PROKR2</i>	c.743G>A	p.Arg248Glu	Third intracellular loop	Polymorphism	Tolerated	Benign

**Table 2** Phenotypes of patients with *FGFR1* and *PROKR2* variants.

Patient	Gene	Sex	Age at diagnosis (years)	Hormonal deficiencies	MRI	Smell test	Other features
I	<i>FGFR1</i>	F	15	GH, LH and FSH	APH, EPP, normal stalk	NL	Japanese ancestry
II	<i>FGFR1</i>	F	4	GH, TSH, LH and FSH	APH, EPP, absent stalk	NL	
III	<i>FGFR1</i>	M	16	GH, TSH, ACTH, LH and FSH	APH, EPP, thin stalk	NL	Micropenis
IV	<i>FGFR1</i>	M	15	GH, TSH, LH and FSH	APH, EPP, absent stalk	NL	Micropenis
V	<i>PROKR2</i>	F	19	GH, ACTH, LH and FSH	NAP, APP absent stalk	NL	Diabetes insipidus
VI	<i>PROKR2</i>	F	10	GH, TSH, partial ACTH, LH and FSH	APH, EPP, absent stalk	NL	Diabetes mellitus, facial asymmetry and clinodactyly

F, female; M, male; APH, anterior pituitary hypoplasia; EPP, ectopic posterior pituitary; NAP, normal anterior pituitary; APP, absent posterior pituitary; NL, normal test.

considering all populations (ALL) of the 1000GENOMES project. Since the patient was born to Japanese parents, it is important to consider the MAF in the Japanese population, which is 2.8%. The *FGFR1* variant p.Pro772Ser has a MAF of 0.5% (ALL 1000GENOMES) and a MAF of 4.2% in African-American population of the Exome Sequencing Project (ESP6500). To date, the *FGFR1* variant p.Arg448Trp has not been described in population databases.

The *PROKR2* variant p.Arg85Cys has been described in the databases of the 1000GENOMES project (MAF=0.2% ALL) and the ESP6500 project (MAF=0.6%) in the African-American population. The *PROKR2* variant p.Arg248Glu has no MAF described in databases. Phenotypes of patients carrying the variants are detailed in Table 2.

#### Patients with *FGFR1* variants

**p.Ser107Leu variant** ► The *FGFR1* variant p.Ser107Leu was identified in a female patient born to non-consanguineous Japanese parents (patient I; Table 2). At first presentation, she was 15.5 years old and had severe short stature ( $-7.6$  s.d.), primary amenorrhoea and delayed bone age (10 years). Endocrine evaluation revealed growth hormone (GH) and LH/FSH deficiencies. She was treated for both deficiencies, gained 15.7 cm and attained a final height of 131.7 cm. Neuroimaging showed a small anterior pituitary, normal stalk and ectopic posterior pituitary. Her father was unavailable for genetic study and her mother was not a carrier of the variant, but her unaffected sister is a carrier of the same variant.

**p.Arg448Trp variant** ► The p.Arg448Trp variant was identified in a 4-year-old girl at first presentation (patient II; Table 2). She was born to non-consanguineous parents, and there was no history of short stature in her family.

The initial complaint was severe short stature ( $-3.0$  s.d.), and endocrine evaluation showed GH and thyroid-stimulating hormone (TSH) deficiencies. Both conditions were treated with good response and the patient achieved the family target height percentile. She had no pubertal signs until 13 years of age, and then puberty was induced with conjugated oestrogens. MRI revealed a small anterior pituitary, absent stalk and ectopic posterior pituitary. Her unaffected father and sister are carriers of the same variant.

**p.Pro772Ser variant** ► We identified the *FGFR1* variant p.Pro772Ser in heterozygous state in two unrelated patients. The first patient (patient III; Table 1) had a history of breech presentation with forceps delivery. He first presented at age 16 years and 9 months with severe short stature ( $-6.7$  s.d.) and lack of sexual development with penile length of  $4.5 \times 1.9$  cm ( $< -2.5$  s.d.), right testes  $1.8 \times 1.0$  cm and left testes  $1.4 \times 0.9$  cm. He had GH, TSH, adrenocorticotrophic hormone (ACTH), LH and FSH deficiencies at laboratory evaluation. He was treated for all deficiencies and achieved a final height of 172.5 cm after 6 years of GH treatment. His unaffected mother is a carrier of the same variant.

The second patient harbouring this variant (patient IV; Table 1) was born at home without health assistance to non-consanguineous parents. At first presentation he was 15 years and 2 months old. The main complaints were short stature ( $-3.5$  s.d.) and lack of sexual development, with penile length  $4.5 \times 1.5$  cm ( $< -2.5$  s.d.), the left testis was retractable and the right testis was  $2.8 \times 1.5$  cm. Endocrine tests confirmed GH, TSH, LH and FSH deficiencies. He was treated for all deficiencies, and GH treatment was carried out for 3.5 years with growth to a final height of 172 cm. At adulthood, he developed obesity class 3 and type 2 diabetes mellitus. His mother is not a carrier of the variant and his father was not available for testing.



## Patients with *PROKR2* variants

**p.Arg85Cys variant** ▶ The p.Arg85Cys variant was identified in a 19-year-old female eunuchoid patient who presented with primary amenorrhoea and no breast development (patient V; Table 2). She was born to non-consanguineous parents. Endocrine tests revealed GH, ACTH, LH and FSH deficiencies. She also developed diabetes insipidus 8 years after the diagnosis of hypopituitarism. Neuroimaging revealed a normal anterior pituitary, absent stalk and a non-visualized posterior pituitary. Intriguingly, this patient reached normal final height (168.3 cm) without GH treatment. Her unaffected father carries the same variant.

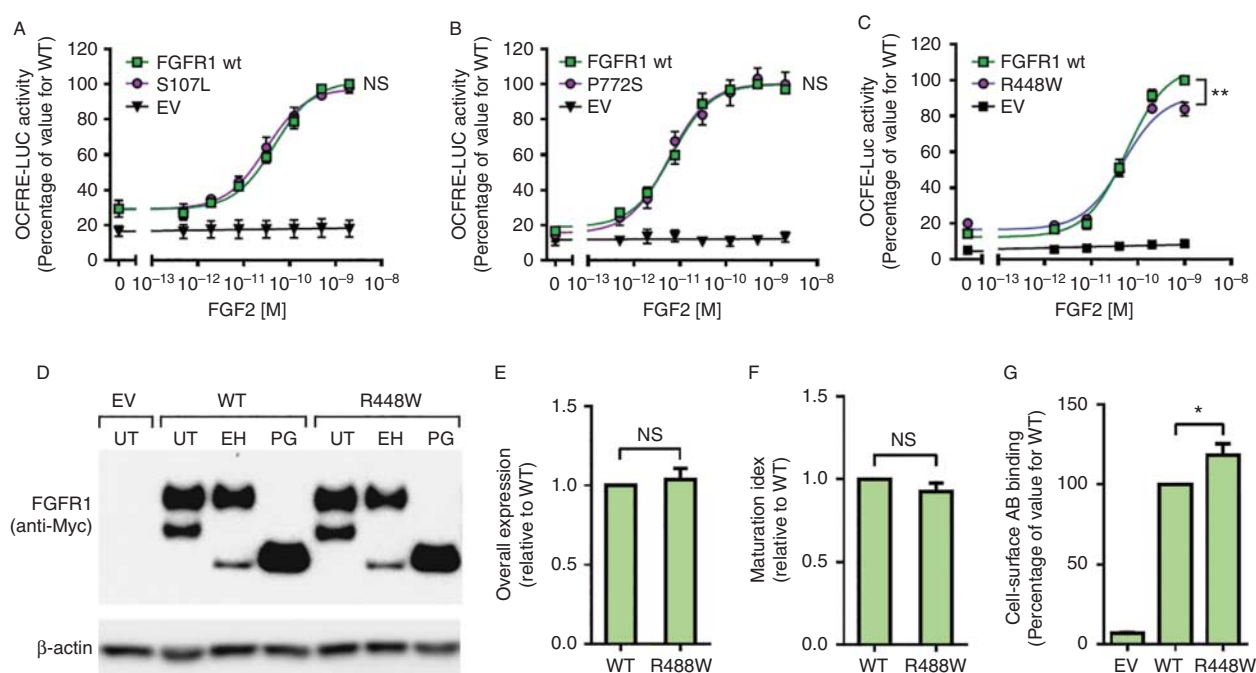
**p.Arg248Glu variant** ▶ The female patient with p.Arg248Glu variant first presented at 10 years of age with severe short stature ( $-5.0$  s.d.; patient VI; Table 2). She was born to non-consanguineous parents at 32 gestational weeks by Caesarean section due to premature membrane rupture. Endocrine evaluation detected GH, TSH, LH, FSH

and partial ACTH deficiencies. MRI revealed a small anterior pituitary, absent stalk and ectopic posterior pituitary. She has some dysmorphic features such as facial asymmetry and clinodactyly. She was treated for 8 years, until 18 years of age, for all deficiencies including GH and achieved a final height of 166.5 cm. At 24 years old, she presented with diabetes mellitus and mild elevation of hepatic enzymes with negative serological test results for hepatitis and negative test results for autoimmune diabetes. Her unaffected mother carries the same variant. Her father died from alcoholic hepatic cirrhosis and had diabetes mellitus along with her brother and several uncles.

All the six patients were tested for olfactory function with The Pocket Smell Test or The Brief Smell Identification Test – Sensonics, Inc. (Haddon Heights, NJ, USA) and had a normal sense of smell.

## FGFR1 functional studies

All the three *FGFR1* variants were predicted to be pathogenic by at least one of the three prediction programmes



**Figure 1**

Functional analysis of *FGFR1* variants. (A, B and C) Signalling activity of WT and altered *FGFR1* receptors in L6 myoblasts. Plotted are means  $\pm$  s.e.m. of three independent experiments. p.Ser107Leu (A) and p.Pro772Ser (B) variants had normal signalling activity, while p.Arg448Trp had reduced maximal signalling activity (\*\*statistically significant ( $P < 0.01$ )). (C) (D) Western blot analysis of CHO cells transiently transfected with empty vector (EV), *FGFR1* WT or p.Arg448Trp constructs. UT, untreated; EH, EndoH

treated; PG, PNGase treated. (E and F) Densitometry analysis of total protein and maturation levels of WT and p.Arg448Trp receptors. Plotted are means  $\pm$  s.e.m. of three independent experiments. (G) Cell-surface expression levels of WT and p.Arg448Trp receptors assessed in live transiently transfected COS-7 cells. Plotted are means  $\pm$  s.e.m. of five independent experiments (\*statistically significant ( $P < 0.05$ )).

tested (Table 1). Signalling activity of these variants was assessed *in vitro* using the well-established FGF-responsive osteocalcin reporter system, which acts downstream of the MAPK pathway. Cells expressing the p.Ser107Leu and p.Pro772Ser variants elicited dose–response curves similar to the WT FGFR1c curve (Fig. 1A and B). The p.Arg448Trp variant, on the other hand, demonstrated a small (approximately 15%) but significant reduction in maximal signalling activity ( $P < 0.01$ ; Fig. 1C). To evaluate potential molecular mechanisms underlying the observed reduced signalling activity, we performed protein expression studies. Overall protein expression and maturation levels of p.Arg448Trp were similar to those for the WT, indicative of normal protein synthesis and folding processes (Fig. 1D, E and F). However, cell-surface expression levels of this variant were significantly increased compared with the WT (approximately 20%,  $P < 0.05$ ; Fig. 1G), indicating a defect in the receptor internalization process.

## Discussion

Congenital hypopituitarism (CH) is implicated in considerable morbidity and leads to premature mortality (18). Although many genetic causes have been discovered, the majority of patients remain without genetic diagnosis (19, 20, 21). In this study, due to the clinical and genetic overlap between IHH/KS and CPHD, we investigated the presence of deleterious variants of two genes classically associated with IHH/KS in a large cohort of Brazilian CPHD patients. We found four patients harbouring three different *FGFR1* variants (p.Ser107Leu, p.Arg448Trp and p.Pro772Ser) and two patients with *PROKR2* variants (p.Arg85Cys and p.Arg248Glu).

## Variants of *FGFR1*

The first detection of *FGFR1* defects in CPHD patients, to our knowledge, was the identification of a submicroscopic deletion including the *FGFR1* gene in a male patient. This patient had GH, LH and FSH deficiencies associated with anosmia and spherocytosis (22). Another submicroscopic deletion involving *FGFR1*, identified during a study analysing 69 Japanese patients with CPHD, was detected in a female patient with CPHD associated with epilepsy, learning disability and Chiari type 1 malformation (23). In addition to the submicroscopic deletion, in this study, two heterozygous missense variants (p.Val102Ile and p.Ser107Leu) were also found in two unrelated patients. These variants were present at a low frequency in the Japanese control group. Results of functional studies of the two variants revealed nearly normal transactivating functions in luciferase assays (Table 3). The *FGFR1* variant p.Ser107Leu was also found in our cohort, in a patient born to Japanese parents, as would be expected, we did not find the variant in our Brazilian control group. The functional study performed by us (Fig. 1) revealed no difference in transactivation in comparison with the WT, confirming the findings of the previous Japanese population study (23). Taking into account the presence of the variant in Japanese controls and population databases, an unaffected first-degree relative carrying the variant, and the *in vitro* data, it is unlikely that this variant (p.Ser107Leu) alone is causing the phenotype.

Concerning the p.Pro772Ser variant, due to its finding in five of the 400 healthy controls, a MAF of 4.2% in African-based populations – one of the major ethnic populations in Brazil – and the presence of an unaffected mother that carries this variant, it is reasonable to conclude that this is a polymorphism.

**Table 3** Characteristics of *FGFR1* missense variants found in patients with CPHD.

Variant	Signalling activity	Expression	Patients		Controls		References
			<i>n</i>	Frequency (%)	<i>n</i>	Frequency (%)	
p.Thr112Thr	NS	NS	103 <sup>a</sup>	3	268	0	(3)
p.Ser450Phe	↓↓	=				0	
p.Pro483Ser	↓↓	=				0	
p.Val102Ile	↓	NS	69	3	100	2	(23)
p.Ser107Leu	=	NS				1	
p.Ser107Leu	=	NS	156	2	400	0	Present study
p.Arg448Trp	↓	NS				0	
p.Pro772Ser	=	NS				1.25	

NS, not studied; =, similar to the WT.

<sup>a</sup>Patients with CPHD and/or SOD.

The p.Arg448Trp variant, on the other hand, has not been described to date in population databases, it shows a reduced signalling activity, probably due to a defect in receptor internalization process (Fig. 1), and is considered deleterious according to three *in silico* prediction tools (Table 1). Taking into account all this information together, it is likely that this variant contributes to the phenotype, although first-degree relatives are unaffected carriers. Also, the possibility of an oligogenic mechanism similar to that seen in IHH/KS (17) cannot be discarded.

In an additional study focusing on the genetic overlap in KS, CPHD and septo-optic dysplasia (SOD), a cohort of 103 patients was examined. Three heterozygous variants (p.Thr112Thr, p.Ser450Phe and p.Pro483Ser) were found in *FGFR1* in three different unrelated patients with CPHD and SOD. The latter two variants showed decreased signalling activity in luciferase reporter assays and similar overall expression and maturation levels in western blotting analysis (3).

In summary, considering the present study and the previously published data for the two other cohorts described above (3, 23), to date, a total of 328 patients with CPHD and/or SOD have been screened for *FGFR1* mutations, and nine patients (2.7%) were found to harbour eight different heterozygous *FGFR1* variants with variable probabilities of having a significant role in the phenotype (Table 3).

### Variants of *PROKR2*

*PROKR2* mutations have been previously screened for in patients with CH. Reynaud and colleagues screened 72 patients with pituitary stalk interruption syndrome (PSIS) and found three heterozygous missense variants (p.Leu173Arg, p.Arg85His and p.Ala51Thr). The first two showed deleterious effects in functional studies supporting, according to the authors, a causative role to the phenotype (24). McCabe and colleagues (25) screened 422 patients with CH, holoprosencephaly and/or SOD and found five missense variants in 11 unrelated patients, three of them (p.Arg85Leu, Leu173Arg and Arg268Cys) proved to be deleterious in functional studies. In the latter study, the p.Leu173Arg variant was inherited by the patient from his unaffected mother, who was proven to be homozygous for the variant. The poor genotype–phenotype correlation led the authors to conclude that the contribution of *PROKR2* variants to the phenotype is uncertain (25).

The *PROKR2* variants found in our cohort of 156 patients have not been previously described in CH, PSIS or SOD, but have already been described in patients with KS/IHH and have been submitted to functional studies

(16, 26, 27). The p.Arg85Cys variant showed modest but a significant 30% reduction in maximal MAPK activation in an Egr1-Luc assay (26). In inositol phosphatidyl (IP) accumulation assays, the variant showed similar dose–response curves to WT *PROKR2*. Also, the p.Arg85Cys variant does not interfere with *PROK2* ligand binding and its plasma membrane expression is not reduced according to the results of western blot analyses (26). Our patient inherited the variant from an unaffected father.

The p.Arg248Glu variant is located within the last intracellular loop, and results of functional analyses revealed normal transcription activity in an Erg-Luc assay but decreased calcium mobilization activity (72% of WT) in an aequorin-based  $\text{Ca}^{2+}$  flux assay. Its expression level was similar to that of the WT *PROKR2* in western blot experiments (16). This variant does not have a MAF described in population databases and the patient's mother is an unaffected carrier.

To date, including our cohort, 650 patients with CPHD and/or SOD have been studied for *PROKR2* mutations and 16 patients (2.5%) harbour eight different heterozygous *PROKR2* variants. Functional studies have been performed on all variants and six of them were proved to be at least partially deleterious.

In conclusion, *FGFR1* and *PROKR2* variants may contribute to the phenotype of patients with CPHD but are unlikely to be implicated in isolation. Consistently with the results of previous studies, we believe that it is likely that other associated genetic and/or environmental factors are involved in the aetiology of this condition.

### Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EC-15-0015>.

### Declaration of interest

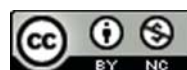
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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